PROJECT No.18CISA093.1

VIRUCIDAL EFFICACY OF A CONCENTRATE PRODUCT AGAINST AFRICAN SWINE FEVER VIRUS (ASFV)

TESTING FACILITY

Instituto Nacional de Investigación y Tecnología Agraria y Alimentaria (INIA)

Carretera de la Coruña Km 7.5, 28040, España

SPONSOR

Virox Technologies Inc.

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Date:

April 08, 2019

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1. PURPOSE

The purpose of this study is to evaluate the virucidal efficacy of a test substance for registration as a virucide. The test procedure is to simulate the way in which the test substance is intended to be used. This method is in compliance with the requirements of and may be submitted to, one or more of the following agencies as indicated by the Sponsor: U.S. Environmental Protection Agency (EPA), Health Canada and Australian Therapeutic Goods Administration (TGA).

2. GLP STATEMENT

No regulatory compliance can be made as the test facility is not a registered GLP facility at the time of conduct of this study. The test facility would endeavor, where possible to conduct the study to GLP principles. The final report will include a section detailing where adherence could not be met.

3. STUDY GUIDELINES

- The study will be performed in compliance with the Quality Management System of INIA.
- All work undertaken will follow the test facility SOPs. In case of any conflict between the requirements of SOP and study protocol, the latest takes priority.
- The study will be conducted using guidelines EPAOCSPP 810.2200 Disinfectants for use on hard surfaces-efficacy data recommendations.

4. TEST SUBSTANCE CHARACTERIZATION

According to 40 CFR, Part 160, Subpart F [160.105] test substance characterization as to identity, strength, purity, solubility and composition, as applicable, will be documented before its use in this study. The stability of the test substance will be determined prior to or concurrently with this study by the sponsor. Pertinent information, which may affect the outcome of this study, will be communicated in writing to the testing facility. The Certificate of Analysis for chemical characterization of each lot of test substance will be appended to the final report.

5. JUSTIFICATION FOR SELECTION OF THE TEST SYSTEM

Regulatory agencies require that a specific virucidal claim for a product intended for use on hard surfaces be supported by appropriate scientific data demonstrating the efficacy of the product (test substance) against the claimed virus. Each agency will accept adequate data generated by any appropriate technique in support of a virucidal efficacy claim. This is accomplished by treating the target virus with the test product under conditions, which simulate as closely as possible, in the laboratory, the actual conditions under which the product is designed to be used. For products intended for use on hard surfaces (dry, inanimate environmental surfaces), a carrier method is used in the generation of the supporting virological data. The African Swine Fever Virus (ASFV) strain BA71V, is a Vero cell adapted strain and will be used in this study to challenge the product. The experimental design in this protocol meets these requirements.

6. SUMMARY OF THE TEST

This test will follow the ASTM E1053- -11 *Virucidal Hard Surface Efficacy Test*. According to this method the virus will be dried on a suitable sterile hard surface at room temperature. The test substance will be

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used to treat the dried virus on a glass Petri dish carrier. After a defined exposure period, the test substance-virus mixture will be neutralized, scraped off from the surface, collected, and tested for the presence of infectious virions.

The proposed starting date of the test is October 7th, 2019 and proposed completion date is October 28th, 2019. Verbal results may be communicated to the sponsor upon completion of the experimental phase, and a written final report will follow.

7. STUDY INFORMATION

This protocol is intended for two lots of the test substance and the main goal of the study is to determine the virucidal activity of a test substance using the specified test conditions.

7.1. Test substance information

Name: Intervention Concentrate

Storage conditions: Room temperature

Lot numbers: 13829 and 13830

Manufacture date: January 23rd, 2019

Expiry date: January 23rd, 2020

Active concentration: Lot 13829 contains 4.02% and Lot 13830 contains 4.01% of Hydrogen Peroxide at ≤LCL

7.2. Test conditions

Dilution: 1:64 defined as 1 part of the test product added to 64 parts of the diluent (AOAC hard water 400ppm)

Test virus: African Swine Fever Virus (strain BA71V)

Contact time: 5 minutes

Exposure Temperature: Room temperature (22-25°C).

Neutralizer: Letheen Broth + 0.1% sodium thiosulfate (final concentration in the medium)

Soil load: A final concentration of 5% fetal bovine serum (FBS) will be added to the virus test suspension.

Application type: Direct liquid application.

7.3. Test virus

The African Swine Virus (ASFV) strain BA71V, is a Vero cell-adapted strain that grows in Vero cells. The original ASF virus was isolated in 1971 from the spleen of an infected animal from Badajoz (Spain). The virus was passaged in swine monocytes. Adaptation of ASF virus (36th passage in swine monocytes) to grow on VERO cells was carried out according to the following infection schedule: 60 mm plastic plates containing 2 to $3x10^6$ cells were incubated with $1.1x10^4$ hemadsorption units (H.A.D.U.) of ASF virus. After

an adsorption period of 2 h at 37 °C, the plates received 5 mL of Dulbecco's modified Eagle medium (DMEM) containing 2% calf serum and they were incubated for one week at 37 °C, with a change of medium after the third day of incubation. At the seventh day the attached cells were removed with trypsin and resuspended in a mixture containing equal parts of the culture media removed after the third and seventh day of incubation, at a concentration of $1x10^6$ cells/mL. A sample of 1 mL of this suspension was added to each one of several plates containing $1x10^6$ uninfected cells/plate. The cell monolayers were trypsinized again after an incubation of one week and the procedure repeated several times until a clear cytopathic effect was apparent. Antiviral screen using strain ASFV BA71V is an excellent model for antiviral studies of ASFV infection.

7.4. Cells

The Vero cell line, originally isolated from African green monkey kidneys was purchased from the American Type Culture Collection (ATTC #CCL81). Vero cells are grown at 37°C in DMEM supplemented with 5% heat-inactivated fetal calf serum (FCS; Invitrogen), 100 IU of gentamicin/mL (Sigma-Aldrich), 2 mM L-glutamine (Invitrogen), and nonessential amino acids (Invitrogen).

7.5. Inoculum preparation

Frozen viral stocks will be thawed on the day of the test. Serum will be added to viral stock to achieve an organic load of 5.0% (if not already 5.0%). If the challenge virus culture is standardized by concentration or dilution, or if a column is used, these manipulations be documented and reported.

Note: A level of approximately 4.8 – 6.8 Log_{10} virus challenge/carrier (as indicated by the plate recovery control load) must be recovered. When cytotoxicity is present, higher levels of viral challenge may be needed to measure a >3 log reduction/carrier.

7.6. Carrier preparation

Prior to use, carriers will be screened, cleaned, and sterilized appropriately according to standard laboratory practice.

For each lot of the test substance, an aliquot of 0.2 - 0.4 mL of stock virus will be spread over an area of approximately 4 in^2 that has been marked on the underside of pre-sterilized 100x150mmm glass Petri dishes. Then the virus will be allowed to dry at ambient temperature (approximately 20 - 30 minutes). The drying time, temperature and relative humidity will be equivalent between the test and control carriers and will be recorded in the final report. The following number of carriers will be prepared:

- One carrier per each lot of test substance.
- One carrier for the plate recovery control.
- One carrier per each lot of test substance for the neutralizer effectiveness/viral interference control
- One carrier per each lot of test substance for cytotoxicity control (using media *in lieu* of virus as the inoculum)

7.7. Test substance preparation

The test substance will be prepared exactly according to the sponsor's directions. If the test substance requires dilution, the diluted test substance should be used for testing within three hours of preparation. The diluted test substance, if not within the test temperature range, will be pre-equilibrated to the test temperature prior to use in the study.

7.8. Test

Two lots of the test substance (liquid) will be tested at one contact time and one replicate (N=1).

For direct liquid application test substance, for each run, after the inoculum has dried, 2.0 mL of the test substance will be added. The dried virus film must be completely covered by the test substance. The plates will remain at the temperature and for the time specified by the sponsor. After the contact period, the test substance will be neutralized with 2.0 mL of appropriate neutralizer and the mixture will be scraped from the surface of the dish with a cell scraper. This post-neutralized sample will be considered approximately a 10^{-1} dilution.

7.9. Infectivity assay

The residual infectious virus in all test and control samples will be detected by viral-induced cytopathic effect (CPE).

Selected dilutions of the post-neutralized samples (test samples) and control samples will be added to cultured host cells (at least four wells per dilution, per sample) and incubated in a CO_2 controlled incubator for 5 days. The inoculated culture will be observed and refed with fresh media as necessary, during the incubation period. These activities, if applicable, will be recorded. The host cells will then be examined microscopically for presence of infectious virus. The resulting virus-specific CPE and test substance-specific cytotoxic effects will be scored (scores from 0 – 4) by examining all test and control samples. These observations will be recorded.

8. CONTROLS

8.1. Plate recovery control (PRC)

This control will be performed in a single run, concurrently with the test substance. The virus inoculum will be spread over the surface of a sterile 100x150mmm glass Petri dish and left to dry at ambient temperature. An equivalent volume of medium *in lieu* of the test substance will be added to the dried virus. Post-contact time, virus will be subjected to the identical neutralization procedure as the test substance. This control will determine the relative loss in virus infectivity resulting from drying and neutralization alone.

The results from this control should confirm recovery of at least 4.8-Log_{10} of infectious virus/carrier in this control, following drying and neutralization. This titer will be used to compare with the titers of the test samples to determine log reduction.

8.2. Neutralizer effectiveness/Viral interference control (NE/VI)

This control will determine if residual active ingredient is present after neutralization and if the neutralized test substance interferes with the virus infection system. This control will be performed for both lots of test substance at one replicate.

The test substance will be processed exactly as the test procedure but *in lieu* of virus inoculum, dried medium will be exposed to the test substance and assayed as previously described. Post-treatment and neutralization, the post-neutralized medium/test substance mixture will be divided into two portions, one for cytotoxicity control and the other for neutralizer effectiveness/viral interference control; and processed as the test.

The neutralizer effectiveness/viral interference control sample will be diluted as follows: using dilution test tubes and appropriate pipette, an aliquot of the post neutralized sample will be used for making serial 10-fold dilutions in medium (for example, 0.5 mL sample + 4.5 mL DM). Following serial dilution, 0.1 mL of a low titered virus, containing approximately 1,000 – 5,000 infectious units of virus, will be added to 4.5 mL of each dilution and held for a period of no shorter than the contact time. Then these samples will be used to inoculate host cells as described for the test procedure.

Selected dilutions of the sample will be added to cultured cell monolayers at a minimum of four wells per dilution per sample, as described in the "Infectivity Assay" (Section 7.9).

8.3. Cytotoxicity control (CT)

This control will be performed for both lots of test substance at one replicate.

The cytotoxicity sample, acquired from the neutralizer effectiveness/viral interference control run, will be diluted and have no virus added. Selected dilutions will be inoculated and incubated in the same manner as the rest of the test and control samples. These effects should be distinct from virus-induced cytopathic effects, which will be evident in the plate recovery control cultures.

8.4. Cell viability control

This control will be performed in a single run. It will demonstrate that cells remain viable throughout the course of the assay period. In addition, it will confirm the sterility of the medium used throughout the assay period. At least four wells of cells will receive only medium and will be incubated and processed with both test and other controls. This will serve as the negative control.

8.5. Virus Stock Titer control

This control will be performed in a single run. An aliquot of the virus used in the study will be directly serially diluted and inoculated onto the host cells to confirm the titer of the stock virus. This control will demonstrate that the titer of the stock virus is appropriate for use and that the viral infectivity assay is performed appropriately.

9. CALCULATION

The 50% tissue culture infective dose per mL (TCID50/mL) will be determined using the method of Reed and Muench (Am. J. of Hyg. 1938, 27:493). These analyses should be described in detail in the final report. The test results will be reported as reduction of the virus titer post treatment with the test article expressed as $\log_{10}$.

- <u>The virus load will be calculated in the following manner:</u>

Virus Load (Log_{10} $TCID_{50}$) = Virus Titer (Log_{10} $TCID_{50}$/mL) + Log_{10} [Volume per sample (mL)]

- The Log_{10} Reduction factor (LRF) will be calculated in the following manner:

Log_{10} Reduction Factor = Initial viral load (Log_{10} $TCID_{50}$ - Output viral load (Log_{10} $TCID_{50}$)

10. TEST ACCEPTANCE CRITERIA

• The infectious virus recovered from the Plate Recovery Control must be ≥ 4.8-log $TCID_{50}$/carrier.

• Viral-induced cytopathic effect must be distinguishable from test substance induced cytotoxic effects (if any).

• Virus must be recovered from the neutralizer effectiveness/viral interference control (not exhibiting cytotoxicity).

• The Cell Viability Control (assay negative control) must not exhibit virus.

11. TEST SUBSTANCE EVALUATION CRITERIA

According to the US Environmental Protection Agency, the test substance passes the test if the following are met:

• The test substance must demonstrate a ≥ 3 log_{10} reduction on each surface in the presence or absence of cytotoxicity; and

• If cytotoxicity is present, the virus control titer should be increased if necessary, to demonstrate a ≥ 3 log_{10} reduction in viral titer on each surface beyond the cytotoxic and neutralized level.

12. STATISTICAL ANALYSIS

A statistical analysis will not be performed on the data derived from this evaluation.

13. THE FINAL REPORT

The final report will include, at a minimum, study and study plan/protocol number, test description, full name and address of the Sponsor, details on the identity and lot number of the test substance, reference substance, if used, and the date of initiation of the testing and date of its completion, identity of the test virus strain, propagation, a description of the methods employed, a summary and analysis of the data, and conclusions as they relate to the objective of the test. In addition to a section detailing where GLP were not met.

14. PROTOCOL CHANGES

Any necessary changes in the approved Study Plan/Protocol will be documented as an amendment, reported to the Sponsor, and added to the final study report. The Sponsor will also be immediately notified of any occurrences that may affect the validity of the study.

15. RECORD RETENTION

All of the original raw data and documentation developed exclusively for this study will be archived at CISA (Carretera Algete-El Casar de Talamanca, Km. 8.1, 28130 Valdeolmos, Madrid) for at least the time required for the product to be registered with the EPA and/or HC. These documents include, but are not limited to:

- Study Protocol, amendments and deviations.
- Raw data for test item and control samples.
- All measured data used in the test.
- Signed copy of the final study report.

16. TEST SUBSTANCE DISPOSITION

It is the responsibility of the Sponsor to retain a sample of the test substance(s) for future audit or evaluation. All unused test material will be disposed of following study completion, unless otherwise indicated by the Sponsor prior to initiation of the study.

REFERENCES

1. Annual Book of ASTM Standards, Section 11 Water and Environmental Technology Volume 11.05 Pesticides, Antimicrobials, and Alternative Control Agents; Environmental Assessment; Hazardous Substances and Oil Spill Response, E1053-11.
2. Flint, J.S., Enquist W.L., Krug M.R., Racaniello R.V. and Skalka M.A. 2000. Principles of virology: molecular biology, pathogenesis and control. Ed. Lippincott Williams and Wilkins, Fourth Edition. Philadelphia, Pa. USA.Reed & Muench calculator. Created November 20, 2004 by Brett D. Lindenbach, PhD.
3. Health Canada, January, 2014. Guidance Document - Disinfectant Drugs.
4. Health Canada, January, 2014. Guidance Document - Safety and Efficacy Requirements for Hard Surface Disinfectant Drugs.
5. Reed, J.L. and Muench, H. 1938. A simple method of estimating fifty per cent endpoints. Am. J. Hyg. Vol. 27 (3): 493-497.
6. U.S. Environmental Protection Agency, Office of Chemical Safety and Pollution Prevention, Product Performance Test Guidelines, OCSPP 810.2000: General Considerations for Uses of Antimicrobial Agents, February, 2018.
7. U.S. Environmental Protection Agency, Office of Chemical Safety and Pollution Prevention, Product Performance Test Guidelines, OCSPP 810.2200: Disinfectants for Use on Hard Surfaces – Efficacy Data Recommendations, February, 2018.
8. Enjuanes, L., A. L. Carrascosa, M. A. Moreno, and E. Vinuela. 1976. Titration of African swine fever (ASF) virus. J. Gen. Virol. 32:471-477.
9. Hakobyan, A., A. Kotsinyan, Z. Karalyan, H. Sahakyan, V. Arakelov, K. Nazaryan, F. Ferreira, H. Zakaryan. 2019. Inhibition of African swine fever virus infection by genkwanin. Antiviral res. 167, 78-82.

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For confidentiality purposes, study information will be released only to the sponsor/representative signing the Study Plan/Protocol (above) unless other individuals are specifically authorized in writing to receive study information.

Other individuals authorized to receive information regarding this study:

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